Exploring new roles for the *rpoS* gene in the survival and virulence of the fire blight pathogen *Erwinia amylovora*

Ricardo D. Santander , Mercedes Monte-Serrano , José J. Rodríguez-Herva , Emilia López-Solanilla , Pablo Rodríguez-Palenzuela & Elena G. Biosca

Abstract

Erwinia amylovora causes fire blight in economically important plants of the family *Rosaceae*. This bacterial pathogen spends part of its life cycle coping with starvation and other fluctuating environmental conditions. In many Gram-negative bacteria, starvation and other stress responses are regulated by the sigma factor RpoS. We obtained an *E. amylovora rpoS* mutant to explore the role of this gene in starvation responses and its potential implication in other processes not yet studied in this pathogen. Results showed that *E. amylovora* needs *rpoS* to develop normal starvation survival and viable but nonculturable (VBNC) responses. Furthermore, this gene contributed to stationary phase cross-protection against oxidative, osmotic, and acid stresses and was essential for cross-protection against heat shock, but nonessential against acid shock. RpoS also mediated regulation of motility, exopolysaccharide synthesis, and virulence in immature loquats, but not in pear plantlets, and contributed to *E. amylovora* survival in nonhost tissues during incompatible interactions. Our results reveal some unique roles for the *rpoS* gene in *E. amylovora* and provide new knowledge on the regulation of different processes related to its ecology, including survival in different environments and virulence in immature fruits.

Keywords
starvation; cross-protection; motility; exopolysaccharides; loquats; incompatible plant–pathogen interactions.

Introduction

Erwinia amylovora is a nonobligate plant pathogenic bacterium that causes fire blight in most species of the subfamily *Maloideae,* of the family *Rosaceae*. Over the last few centuries, this disease has spread all over the world, causing serious economical losses in places where susceptible hosts are cultivated (Kamber *et al.*, 2012). Fire blight affects fruit trees such as apple (*Malus domestica*), pear (*Pyrus communis*), loquat (*Eriobotrya japonica*), or quince (*Cydonia oblonga*), and also a variety of ornamental species of the genera *Sorbus, Crataegus, Pyracantha*, and *Cotoneaster,* among others. The success of *E. amylovora* dissemination is related to the ability of this enterobacterial phytopathogen to survive under unfavorable conditions and to spread through different means, including insects, birds, rainwater, and contaminated pruning tools (Thomson, 2000; van der Zwet *et al.*, 2012). Accordingly, *E. amylovora* spends part of its life cycle facing starvation until it finds new susceptible hosts where it can multiply again.

As in the case of *E. amylovora,* it is accepted that bacterial pathogens outside their hosts are mostly exposed to the oligotrophic conditions prevailing in natural environments, and rarely finding a nutrient source allowing active growth for prolonged periods (Morita, 1997; Edwards, 2000). Thus, the perdurability of pathogens in nature is related to their ability to survive to nutrient scarcity, a stress that bacteria also experience during the entry into the stationary growth phase in batch culture conditions. In *Escherichia coli* and other bacterial species, the main regulator during the adaptation of cells to starvation is the alternative sigma factor of the RNA polymerase RpoS, encoded by the gene *rpoS* (Ishihama, 1997; Hengge-Aronis, 2002; Navarro Llorens *et al.*, 2010). Genes homologous to the *E. coli rpoS* gene have been identified and characterized in a variety of bacterial species pertaining to the branch gamma of the

proteobacteria (Dong & Schellhorn, 2010). The sigma factor RpoS controls different physiological processes, and it is induced not only by starvation but also by other stresses and during the entry into the stationary growth phase, acting as the master regulator of the general stress response. As a result, starved and stationary phase cells become protected against multiple stresses to which they were not exposed, in a phenomenon called cross-protection (Hengge-Aronis, 2002). In the case of bacterial pathogens, this cross-protection might not only be important for their survival outside the host, but also during the infectious process, when they are exposed to host defenses and other stresses inside plant tissues. In this regard, the virulence and/or pathogenicity of many bacterial species are also regulated by RpoS, via either the direct control of virulence/pathogenicity factors or indirectly, modulating the expression of virulence-related genes that enhance the biological fitness of the pathogen in the environment (Dong & Schellhorn, 2010).

In a previous work, Santander *et al.* (2014) monitored the regulation of the *rpoS* gene during *E. amylovora* exposure to starvation and characterized two physiological mechanisms developed by this pathogen to deal with this stress, the starvation survival and the viable but nonculturable (VBNC) responses. Both are characterized by cell growth arrest, but the latter implies the inability of viable cells to form colonies on solid media. With the purpose of investigating the role of *rpoS* on these survival strategies, an *rpoS* mutant of the European reference *E. amylovora* strain CFBP 1430 and a complemented strain were obtained, and the effects of the *rpoS* impairment on culturability, viability, and integrity of starved cells over time were evaluated. We additionally explored other functions of the sigma factor RpoS in *E. amylovora,* some of which have not yet even been studied in other pathogens. First, we determined the contribution of *rpoS* to stationary phase cross-protection against different stresses. Given that RpoS controls virulence/pathogenicity in many pathogens, we also evaluated its role in the control of *E. amylovora* virulence and pathogenicity factors, such as motility or the synthesis of the two main exopolysaccharides, levan and amylovoran. Finally, we assessed the effect of the *rpoS* mutation on *E. amylovora* virulence in different types of susceptible host plant material, as well as its potential role during incompatible plant–pathogen interactions.

Materials and methods

Bacterial strains, vectors, and culture conditions

The bacterial strains and plasmids used in this study are shown in Table 1. Bacterial cells were cryopreserved in 25% (v/v) glycerol at −80 °C. For most experiments, *E. amylovora* and *E. coli* strains were grown on Luria–Bertani (LB) agar plates or broth with shaking (200 r.p.m.) at 28 °C or at 37 °C, respectively. Late stationary phase ($OD_{600\ nm} > 2$) and mid-log phase ($OD_{600\ nm} = 0.5$) cultures were obtained as follows. Cells were grown overnight in LB, diluted 1/100 into fresh medium, and incubated at 28 °C with shaking for 16 h in the case of stationary phase cultures, or *c.* for 6 h in the case of mid-log phase cultures. In some experiments, *E. amylovora* cells were grown in modified basal medium A (MBMA) (per L, 3 g KH_2PO_4, 7 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 2 mL glycerol, 0.5 g citric acid, 0.03 g $MgSO_4$) (Torriani, 1960) supplemented with 100 μg L^{-1} nicotinic acid (Starr & Mandel, 1950) (MBMANic). When necessary, antibiotics were added to the media at the following final concentrations: kanamycin 20 μg mL^{-1} (Km^{20}), ampicillin 100 μg mL^{-1} (Ap^{100}), and chloramphenicol 10 μg mL^{-1} (Cm^{10}).

Obtaining and complementation of an *E. amylovora* mutant in the *rpoS* gene

Primers used in this study (Table 2) were designed based on the genome sequence of the European reference *E. amylovora* strain CFBP 1430 (Smits *et al.*, 2010) (WT1, Table 1), available in the ASAP database, https://asap.ahabs.wisc.edu (Glasner *et al.*, 2003). To obtain an *rpoS* mutant, a DNA fragment containing the *E. amylovora rpoS* gene was amplified by PCR, cloned into the pGEM-T Easy Vector System I (Promega, Madison, WI), digested with BamHI, and interrupted with the kanamycin resistance interposon Ω-Km from pHP45Ω-Km (Table 1). The constructed vector pGEM*rpoS*::Ω-Km was introduced into strain CFBP 1430 by electroporation, and marker exchange mutagenesis was carried out using the Roeder & Collmer (1985) low-phosphate medium. Given that *rpoS* mutants of many bacterial species are sensitive to hydrogen peroxide (H_2O_2), *rpoS* mutations were phenotypically confirmed by a growth inhibition halo assay. Briefly, overnight cultures in LB were washed with SS and plated onto MBMANic agar. Then, a sterile paper filter disk was placed on seeded plates, 5 μL of 33% (v/v) H_2O_2 loaded onto each disk, and the plates were incubated for 48 h at 28 °C. Afterward, the correct disruption of the *rpoS* gene was verified by PCR (Table 2) and DNA sequencing.

To obtain a complemented strain, a functional copy of *rpoS* was amplified by PCR (Table 2), cloned into the XmaI–SpeI site in the broad-host-range plasmid pBBR1MCS-4 (Table 1), and the resulting construct electroporated into the mutant strain *rpoS*$^{-}$. The mutant complementation was confirmed phenotypically by the growth inhibition halo assay, as previously described. Then, both the inactivation and complementation of the

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. amylovora		
CFBP 1430	Wild-type strain 1, WT1; isolated from *Crataegus* sp. in France	Paulin & Samson (1973)
rpoS^{-}	Mutant strain derived from CFBP 1430; *rpoS*::ΩKm; Km^{r}	This study
rpoS^{+}	Complemented mutant strain containing a functional copy of *rpoS* in pBBR4::*rpoS;* Km^{r}, Ap^{r}	This study
Ea 1/79	Wild-type strain 2, WT2; isolated from *Malus sylvestris* in Germany	Falkenstein *et al.* (1988)
Ea 1/79Sm-del100	Mutant strain derived from Ea 1/79 containing a deletion from *ams*A to *ams*F; deficient in amylovoran synthesis; Cm^{r}; Amy^{-}	Bugert & Geider (1995)
Ea 1/79-18M	Mutant strain derived from Ea 1/79 lacking levansucrase activity; *rls*B::pfdA8; Km^{r}; Lev^{-}	Du & Geider (2002)
E. coli		
DH5α	*Sup*E44Ω*lac*U169 (80*lacZ*ΩM15) *hsdR*17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*-A1	Hanahan (1983)
Plasmids		
pGEM-T	SP6; T7; lacZ; Ap^{r}	Promega
pGEM*rpoS*	pGEM-T containing a 1047-bp PCR product with the wild-type *E. amylovora* (CFBP 1430) *rpoS* gene; Ap^{r}	This study
pGEM*rpoS*::Ω-Km	pGEM*rpoS* containing the Ω-Km interposon from pHP45Ω-Km in the unique BamHI site inside the *rpoS* gene; Ap^{r}; Km^{r}	This study
pHP45Ω-Km	Plasmid containing the interposon Ω-Km; Ap^{r}, Km^{r}	Fellay *et al.* (1987)
pBBR1MCS-4	Broad-host-range expression vector; RK2; lacZ; Ap^{r}	Kovach *et al.* (1995)
pBBR4::*rpoS*	pBBR1MCS-4 containing a functional copy of the *E. amylovora* (CFBP 1430) *rpoS* gene in the XmaI–SpeI restriction site	This study

Table 2. Primers used in this study

Primers	Sequence
rpoS amplification	
rpoS5′	ACCTTGCGGTTCTGCCTTG
rpoS3′	GTTCTTCGCGCTATCATTCACGGAAG
Mutagenesis confirmation by PCR	
*rpoS*_EXT.F	GAAGGTGGCAATAAAGGTATCG
*rpoS*_EXT.R	GTAGTGCTGCCAGTACCAAA
T4-Ω	AGCTTGCTCAATCAATCACCG
Mutagenesis confirmation by RT-PCR	
RT-*rpoS*.F	AAGATTGCCCGACGTTACAG
RT-*rpoS*.R	AGACGAATGGTACGGGTTTG
Mutant complementation	
*rpoS*_XmaI.F*	AAATTAA**CCCGGG**ACCTTGCGGTTCTGCCTTG
*rpoS*_SpeI.R†	CCAAGC**ACTAGT**TTCGCGCTATCATTCACGG

*Bold nucleotides indicate the restriction site for XmaI; underlined nucleotides are a clamp sequence.

†Bold nucleotides indicate the restriction site for SpeI; underlined nucleotides are a clamp sequence.

rpoS gene were additionally corroborated by endpoint reverse transcriptase (RT)-PCR (Table 2).

To characterize the mutant and complemented strains, a biochemical profile was determined using the API 20E and API 50CH systems (BioMérieux). Gelatin hydrolysis was additionally tested by a gelatin plate method, according to Smith & Goodner (1958), using 4% (w/v) gelatin as the substrate and an incubation period of 7–14 days at 28 °C.

Oligotrophic microcosms preparation and analysis of population cell dynamics

To determine the role of *rpoS* in *E. amylovora* responses to starvation, natural water microcosms were prepared similar to Santander *et al.* (2012, 2014). Briefly, *E. amylovora* cultures of strains CFBP 1430, *rpoS*$^{-}$, and *rpoS*$^{+}$ were grown overnight in LB and washed twice with sterile natural water. Thereafter, bacterial suspensions were adjusted to an $OD_{600\ nm}$ of 1.0 (about 10^{9} CFU mL^{-1}) and diluted 1/100 into natural water microcosms, reaching *c.* 10^{7} CFU mL^{-1}. Microcosms were incubated at 28 °C. Population dynamics were monitored for 12 days, estimating the number of culturable, viable, and total cells throughout this period according to Santander *et al.* (2012, 2014). This experiment was repeated twice, with three technical replicates in each independent assay.

Stationary phase cross-protection assays

To study the contribution of the *rpoS* gene to stationary phase cross-protection against oxidative, osmotic, and acid stresses, and also against heat and acid shock, late stationary phase and mid-log phase cultures of *E. amylovora* strains CFBP 1430, *rpoS*$^{-}$, and *rpoS*$^{+}$ were exposed to each stress and the behavior of culturable cells compared. For this purpose, late or mid-log phase cultures were washed thrice in sterile saline (SS), diluted to

10^6 CFU mL^{-1}, and subjected to the above-mentioned stresses in 1.5 mL microcentrifuge tubes placed in a dry bath preheated to the assay temperature. Unless otherwise indicated, stresses were assayed at 28 °C. All the incubation periods and conditions employed for each stress were previously optimized in initial assays (data not shown). Changes in the culturability of *E. amylovora*-challenged cells were monitored throughout time by drop plate on LB agar.

The assayed stresses were selected based on other publications with *rpoS* mutants and/or some aspects related to the ecology of *E. amylovora*. Oxidative stress was assayed for 9 min in SS containing 25 mM H_2O_2. Heat shock was assessed at 42 °C in SS for 25 min. Osmotic stress was evaluated for 60 min using artificial nectar (25.5% free sugars) composed of a mixture 5.5 : 4.5 of fructose and glucose in 10 mM phosphate buffer (PB) pH 7.0. The selected sugar composition was based on that present in pear nectar, and the sugar concentration can be present both in pear and in apple nectar (Farkas *et al.*, 2004; Konarska *et al.*, 2005; Spinelli *et al.*, 2005). Acid stress and acid shock were analyzed using PB pH 5.5 for 48 h and PB pH 3.5 for 30 min, respectively. All the assays were performed in at least three independent experiments.

Swimming motility assays

Swimming motility was determined on semisolid LB medium containing 0.3% (w/v) agar, supplemented with antibiotics when required. Overnight cultures of *E. amylovora* strains CFBP 1430, *rpoS*$^-$, and *rpoS*$^+$ were pelleted by centrifugation, and these pellets were used to inoculate plates with a sterile toothpick. Motility plates were incubated at 22 °C (Raymundo & Ries, 1981) for 48 h and photographed with a digital camera (Spectracore Inc., Ontario, NY). Motility areas were determined with IMAGEJ software (Schneider *et al.*, 2012). Swimming motility was assessed in three independent experiments with 10 technical replicates in each case.

Quantification of amylovoran and levansucrase activity

Amylovoran was measured by the cetylpyridinium chloride (CPC) method, according to Edmunds *et al.* (2013). Briefly, *E. amylovora* strains CFBP 1430, *rpoS*$^-$, *rpoS*$^+$ as well as the positive and negative control strains Ea 1/79 (WT2) and Amy$^-$ (Table 1), respectively, were grown overnight in MBMANic plus 1% (w/v) sorbitol, 1/100 diluted in the same medium, and incubated for 40 h at 28 °C with shaking (200 r.p.m.). Then, the $OD_{600\ nm}$ of bacterial suspensions was measured, cells pelleted by centrifugation, and supernatants filtered-sterilized through a pore size of 0.2 μm. Amylovoran was quantified after mixing 0.8 mL of supernatant with 40 μL of 50 mg mL^{-1} CPC. The turbidity of amylovoran was measured at an $OD_{600\ nm}$ after an incubation period of 10 min at room temperature. Amylovoran production was determined in three independent experiments with, at least, six technical replicates in each one.

The secreted levansucrase activity was measured according Ordax *et al.* (2010). Briefly, overnight cultures in Standard I Nutrient Broth (Merck) of *E. amylovora* strains CFBP 1430, *rpoS*$^-$, *rpoS*$^+$, and the positive and negative control strains Ea 1/79 and Lev$^-$ (Table 1), respectively, were 1/100 diluted into fresh medium, incubated at 28 °C (200 r.p.m.) for 20 h, and the $OD_{600\ nm}$ of cells measured (see below). Afterwards, cells were removed by centrifugation, supernatants mixed 1 : 1 with assay buffer (50 mM Na_2HPO_4, 2 M sucrose, 0.05%, w/v, sodium azide), and the turbidity caused by levan formation measured at $OD_{580\ nm}$, after 24 h of incubation at 28 °C. Secreted levansucrase activity was determined in three independent experiments performed in triplicate. Levan production was also qualitatively determined by comparison of colony sizes on Sucrose Nutrient Agar (SNA) plates (EPPO, 2013).

Quantitative data were normalized dividing the $OD_{600\ nm}$ or $OD_{580\ nm}$, respectively, corresponding to amylovoran or levan quantification, by the $OD_{600\ nm}$ of the corresponding analyzed culture.

Virulence assays in immature loquats and pear plantlets

The role of *rpoS* on the *E. amylovora* virulence was evaluated in immature loquats (*E. japonica* cv. Tanaka) and in 4-week-old pear plantlets (*P. communis* cv. Passe Crassane) similar to Santander *et al.* (2014). Briefly, fruits were surface-disinfected for 5 min with 2% (w/v) NaOCl and thoroughly washed with sterile distilled water. Disinfected loquats were inoculated in a single wound (0.5 cm deep) performed with a sterile 100-μL pipette tip. Pear plantlets were inoculated in the wound resulting from the removal of the upper part (about 1 cm) of the stem. To prepare bacterial inocula, 1 mL overnight cultures were washed thrice in SS and their $OD_{600\ nm}$ adjusted to 1.0. Then, cell suspensions were serially 10-fold-diluted in SS and fruits and plantlets inoculated with 2 μL of 10^7, 10^6, or 10^5 CFU mL^{-1}, corresponding to about 10^4, 10^3, and 10^2 CFUs per wound. Each inoculum dose was assayed in 11 fruits and 10 plantlets, in two independent assays. Negative controls were inoculated with SS. Immature fruits and pear plantlets were incubated under controlled conditions and monitored for fire blight symptom

development. To confirm results, *E. amylovora* was re-isolated from fruits and plants showing fire blight symptoms and identified by molecular procedures, following the protocols of the European and Mediterranean Plant Protection Organization (EPPO, 2013).

Hypersensitive response (HR) elicitation and survival assays in nonhost tissues

To determine the contribution of RpoS to the elicitation of the HR and the survival of *E. amylovora* inside nonhosts during incompatible interactions, tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants grown under greenhouse conditions were employed. Overnight cultures of *E. amylovora* strains CFBP 1430, *rpoS*$^{-}$, and *rpoS*$^{+}$ in LB broth were washed thrice in SS and cell densities adjusted to an $OD_{600\ nm}$ of 1.0. Tobacco leaves were infiltrated with about 25 μL of bacterial suspension, using a needleless syringe. Each strain was inoculated twice per leaf, in two leaves per plant, in a total of six plants. Negative controls were inoculated with SS. Infiltrated plants were incubated in the laboratory for 8 days at room temperature, inside a biosafety cabinet. To determine the survivability of inoculated strains in nonhost tissues, two inoculated leaves were processed at times 0, 1, 2, 3, 5, and 8 days as follows. Inoculated leaves were rinsed with sterile distilled water. Then, challenged tissues were cut with sterile scissors and photographed, and infiltrated areas were measured with IMAGEJ software (Schneider *et al.*, 2012). Afterward, each leaf section was homogenized with a sterile micropistil in 0.2 mL antioxidant maceration buffer (AMB) (Gorris *et al.*, 1996) and serially 10-fold-diluted in SS. The number of culturable cells was determined by drop plate on CCT semi-selective medium (Ishimaru & Klos, 1984). Results were represented as CFUs cm^{-2} of infiltrated area analyzed. This experiment was performed twice, in two independent repeats. The ability of *rpoS*$^{-}$ and *rpoS*$^{+}$ to grow on CCT agar plates and to survive in AMB was confirmed in preliminary experiments (data not shown), and no differences were observed when compared to the wild-type strain during the assayed periods.

Statistical analysis

For the statistical analysis, data of *E. amylovora* culturable, viable, and total cell counts from independent experiments were normalized by logarithmic transformation. In the case of percentages, data were normalized by arcsine transformation prior to the analysis. Null data from culturable counts below the detection limit were excluded from statistical analysis. Statistical significance of differences was determined by two-way ANOVA followed by Bonferroni or Dunnett's post *hoc* tests. $P < 0.05$ were considered significant.

Results

Construction and characterization of the *E. amylovora rpoS* mutant and the complemented strain

The mutation of the *E. amylovora rpoS* gene was carried out by homologous recombination with a copy of the wild-type gene interrupted with the Ω-Km interposon (Supplementary Information, Fig. S1a), as described in the materials and methods section. Double recombinants were confirmed by PCR (Fig. S1b) and DNA sequencing. The obtained PCR amplicons (Fig. S1b) coincided with those expected (Fig. S1a) in each strain, and the sequencing data confirmed the interruption of the *rpoS* gene with the Ω-Km interposon in the *rpoS* mutant (data not shown). The complemented strain was obtained transforming the mutant *rpoS*$^{-}$ with a plasmid containing a wild-type copy of the *E. amylovora rpoS* gene. The RT-PCR analysis revealed the presence of *rpoS* transcripts in both the wild-type and the complemented strains, but not in the mutant strain, confirming the interruption of *rpoS* also at the transcription level (Fig. S1c). The *E. amylovora rpoS* mutation and complementation were phenotypically confirmed by the growth inhibition halo assay in the presence of H_2O_2 (Fig. S2). After 48 h of incubation at 28 °C the wild-type strain developed halos of about 3 cm^2, while the *rpoS* mutant showed an increased sensitivity to oxidative stress, with halos of 12 cm^2. The restoration of the parental phenotype in the complemented strain was evidenced by halos of about 1.2 cm^2, slightly smaller than those observed in the wild-type strain.

The *E. amylovora* strains CFBP 1430, *rpoS*$^{-}$, and *rpoS*$^{+}$ showed nearly the same biochemical profiles in API 20E and API 50CH systems, except for a reduced gelatinase activity of the *rpoS* mutant in the gelatinase test of the API 20E system (data not shown), which was confirmed in the gelatin plate method (Fig. S3).

Erwinia amylovora rpoS mutant fails to develop normal starvation responses

Population dynamics of *E. amylovora* strains CFBP 1430, *rpoS*$^{-}$, and *rpoS*$^{+}$ exposed to nutrient limitation in natural water at 28 °C are shown in Fig. 1. During the first 7 days, the wild-type strain (Fig. 1a) slightly decreased culturable cell numbers from 10^7 to 10^6 CFU mL^{-1}, developing a typical starvation survival response. In the following 5 days, a more pronounced entry of culturable cells into the VBNC state was observed, with a drop from

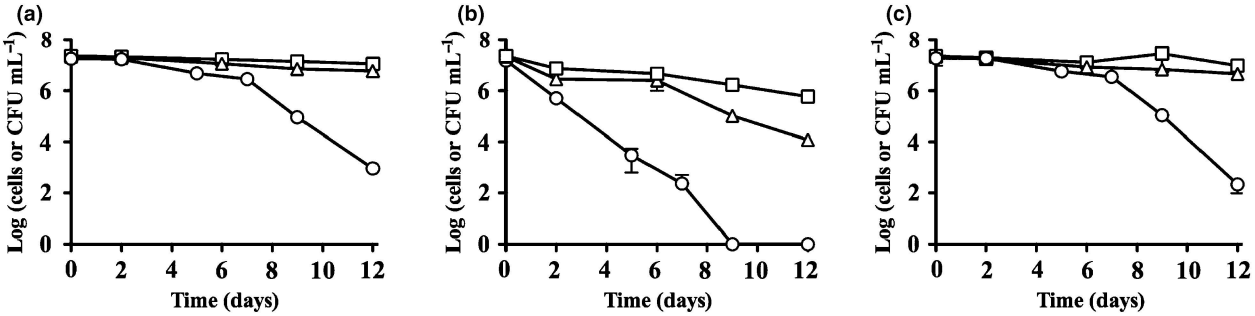

Fig. 1. Role of *rpoS* on *Erwinia amylovora* starvation responses in natural water microcosms at 28 °C. Strains represented are CFBP 1430 (a), *rpoS*$^{-}$ (b), and *rpoS*$^{+}$ (c). Total, viable, and culturable cell counts are indicated with squares, triangles, and circles, respectively. Data shown correspond to the average value of two independent experiments with three technical replicates. Bars represent the SD.

10^6 to 10^3 CFU mL^{-1}, while viable and total cell counts were maintained at numbers similar to the initial ones, about 10^7 cells mL^{-1}. The mutant *rpoS*$^{-}$ (Fig. 1b) did not develop the starvation survival response observed in the parental strain (Fig. 1a). By contrast, it showed a continuous loss of culturability throughout time ($P < 0.001$), with colony counts below the detection limit (1 CFU mL^{-1}) 9 days after the inoculation. Unlike the parental strain (Fig. 1a), the *rpoS*$^{-}$ strain also showed a drop in viable (from about 10^7 to 10^4 cells mL^{-1}) ($P < 0.001$) and total cell numbers (from about 10^7 to 10^5 cells mL^{-1}) ($P < 0.01$) throughout the experiment, these being higher than culturable cell counts (Fig. 1b). Thus, the *E. amylovora rpoS* mutant (Fig. 1b) entered the VBNC state faster than the wild-type strain during the exposure to starvation. Furthermore, the faster adoption of this strategy was accompanied by a loss of viability and cell integrity not observed in the parental strain. In the case of the complemented strain *rpoS*$^{+}$ (Fig. 1c), culturable, viable, and total cell population dynamics were very similar to those observed in the parental strain (Fig. 1a).

RpoS is necessary for full *E. amylovora* stationary phase cross -protection against several stresses

Results corresponding to the stationary phase cross-protection assays in *E. amylovora* strains CFBP 1430, *rpoS*$^{-}$, and *rpoS*$^{+}$ are represented in Fig. 2. The *rpoS* mutant was more sensitive ($P \leq 0.01$) than the parental strain to oxidative stress (Fig. 2a), heat shock (Fig. 2b), osmotic stress (Fig. 2c), and acid stress at pH 5.5 (Fig. 2d), regardless of the growth phase analyzed. By contrast, no differences among strains were observed when exposed to acid shock (Fig. 2e). The *rpoS*-mediated protection of log phase cells was especially significant ($P < 0.0001$) for osmotic (Fig. 2c) and acid stress (Fig. 2d). In the case of stationary phase cells, the protection due to *rpoS* was similar for all the stresses ($P < 0.0001$) (Fig. 2a, b, c and d). Furthermore, significant differences between log and stationary phase cells of the three assayed *E. amylovora* strains were detected in most of the stresses assayed ($P \leq 0.01$), stationary phase cells being more stress resistant than log phase cells (Fig. 2a, c, d and e). However, in the case of heat shock (Fig. 2b), differences between log and stationary phase cells were only significant in the parental strain ($P \leq 0.05$), but not in the *rpoS* mutant. The complemented strain *rpoS*$^{+}$ behaved similarly or survived better than the parental strain when exposed to the stresses analyzed (Fig. 2).

Erwinia amylovora rpoS mutant fails to develop a normal swimming motility

Results of swimming motility assays are summarized in Fig. 3. After an incubation period of 48 h at 22 °C, the three assayed *E. amylovora* strains showed different sizes of swimming areas. The wild-type strain developed swimming halos of 7.1 ± 1.0 cm^2 SD, while the *rpoS* mutant reached 4.7 ± 0.8 cm^2 SD ($P < 0.0001$). The complemented strain *rpoS*$^{+}$, with a swimming halo of 8.1 ± 0.8 cm^2 SD, showed increased motility with respect to the mutant *rpoS*$^{-}$ ($P < 0.0001$), and also to the parental strain ($P < 0.001$). These results reveal the necessity of *rpoS* for *E. amylovora* to develop normal swimming motility.

Erwinia amylovora rpoS mutant shows altered exopolysaccharides production

The relative quantification of amylovoran and levansucrase activity in *E. amylovora* strains CFBP 1430, *rpoS*$^{-}$, and *rpoS*$^{+}$, as well in the positive (Ea 1/79) and negative (Amy^{-}, Lev^{-}) controls, is shown in Fig. 4. The *rpoS* mutant strain produced 1.2 times less amylovoran than the parental strain, this small difference being statistically significant ($P < 0.05$) (Fig. 4a). The complemented strain showed amylovoran levels 1.2 times above those observed

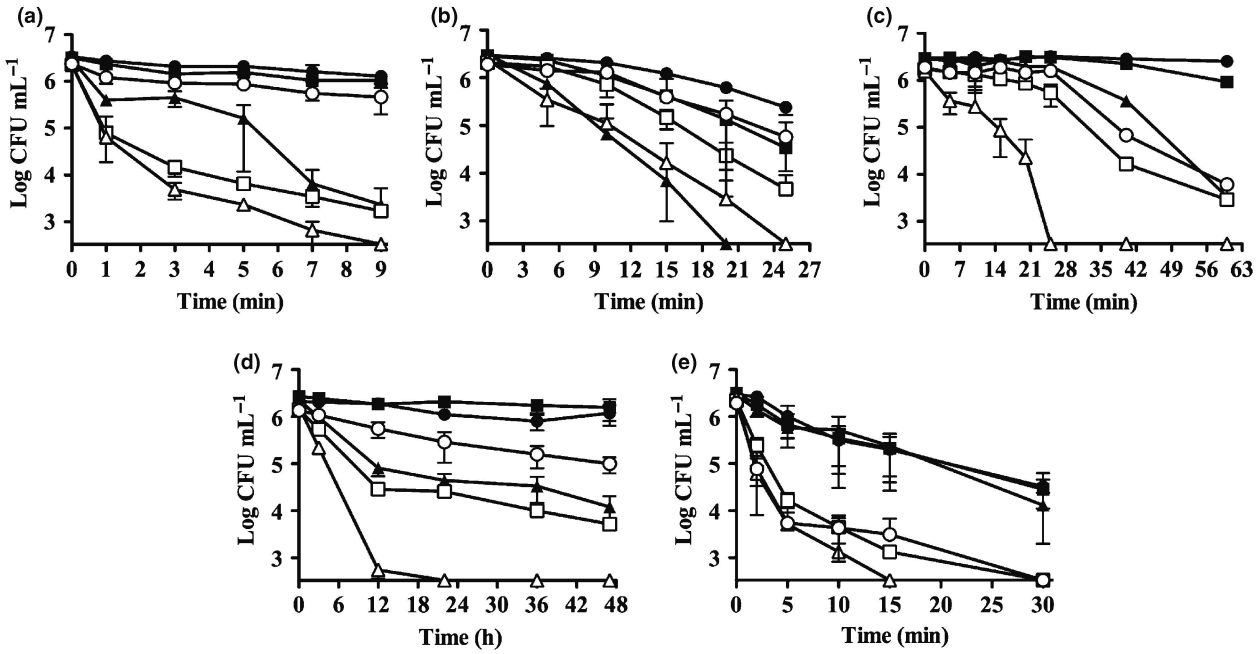

Fig. 2. Role of *rpoS* on the *Erwinia amylovora* stationary phase cross-protection against oxidative stress (a), heat shock (b), osmotic stress (c), acid stress (d), and acid shock (e), using strains CFBP 1430 (squares), *rpoS*$^{-}$ (triangles), and *rpoS*$^{+}$ (circles). Stationary and log phase cells are represented with filled and empty symbols, respectively. Represented data corresponds to the mean of at least three independent experiments. Bars show the SD.

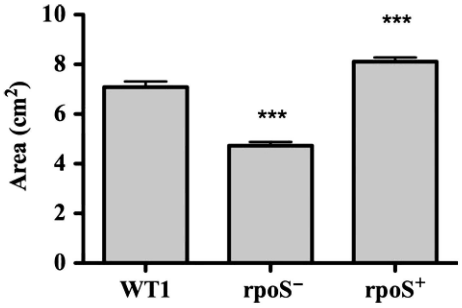

Fig. 3. Effect of *rpoS* mutation on the *Erwinia amylovora* swimming motility. Data shown correspond to the average value of three independent assays after 48 h of incubation at 22 °C and with, at least, 10 technical replicates. Bars represent the SD. The three asterisks indicate statistically significant differences with the wild-type strain, with a *P* value below 0.001. WT1, *E. amylovora* strain CFBP 1430.

in the parental strain ($P < 0.01$). The positive control strain Ea 1/79 showed levels of amylovoran similar to CFBP 1430, and the amount of amylovoran in the negative control Amy^{-} was near 0.

The secreted levansucrase activity in the *rpoS* mutant was about 2.1 times higher than that observed in the parental strain CFBP 1430 ($P < 0.0001$) (Fig. 4b). Levansucrase activity in the complemented strain was 2.2 times lower ($P < 0.0001$) than that in the parental strain, confirming the repression of levan production exerted by *rpoS*. The positive (Ea 1/79) and negative (Lev^{-}) control strains displayed levansucrase levels slightly lower than the wild-type strain CFBP 1430 and near 0, respectively. These phenotypes were additionally confirmed on SNA plates, where colonies of *rpoS*$^{-}$ produced more levan than those of the parental strain (data not shown).

rpoS mutation affects *E. amylovora* virulence in immature fruits but not in pear plantlets

Results from the virulence analysis of the *E. amylovora* strains CFBP 1430, *rpoS*$^{-}$, and *rpoS*$^{+}$ in immature loquats and in pear plantlets are shown in Figs. 5 and 6. In immature fruits, the three assayed strains showed similar time lapses between their inoculation and the onset of fire blight symptoms. However, the *rpoS* mutant was able to cause necrotic lesions in a greater percentage of fruits throughout time, differences with the parental strain being statistically significant at 2 dpi ($P < 0.01$) in fruits inoculated with 10^4 CFUs (Fig. 5a), or at 3 dpi ($P < 0.01$) in those challenged with 10^3 CFUs (Fig. 5b). In fruits inoculated with 10^2 CFUs (Fig. 5c), results were more variable and no significant differences were detected among the assayed strains.

The analysis of the necrotic areas over time in immature loquats inoculated with 10^3 CFUs per wound

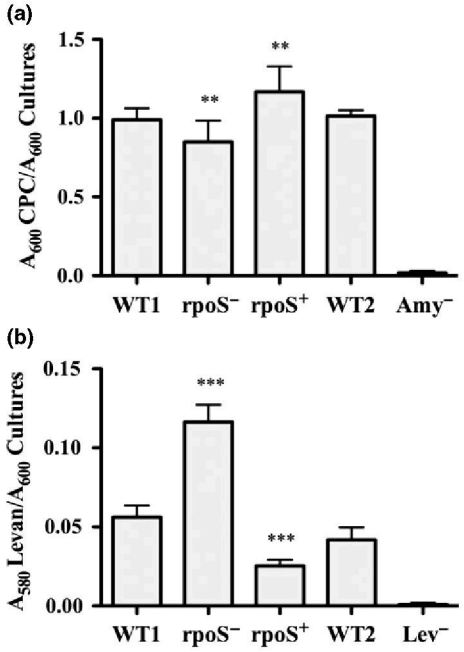

Fig. 4. Effect of *rpoS* mutation on amylovoran production (a) and levansucrase activity (b). In the case of amylovoran quantification, represented data correspond to the mean of three independent repeats, with at least six replicates. Levansucrase activity was measured on three occasions, in triplicate. Bars correspond to the SD. Asterisks denote statistically significant differences with the wild-type strain (**$P < 0.01$; ***$P < 0.001$). WT1 and WT2, *Erwinia amylovora* strains CFBP 1430 and Ea 1/79, respectively.

(Fig. 6a and b) revealed a greater ability of *rpoS*$^-$ to necrose tissues with respect to the parental strain. Necrotic areas of the *rpoS* mutant were about 2.2 times larger than those of the wild type at 5 dpi ($P < 0.001$), and 1.7 times at 7 dpi ($P < 0.001$). No statistically significant differences were observed between the parental and the complemented strain in any of the postinoculation periods analyzed (Fig. 6a and b).

The virulence analysis using pear plantlets and different bacterial doses revealed no differences among strains, either in the onset of symptoms, the number of plants showing fire blight symptoms at a given time (data not shown), or in the intensity of exudates and/or necrosis in inoculated plants (Fig. 6c). Negative controls did not show any of the above-mentioned symptoms in any of the periods assayed (Fig. 6c).

Erwinia amylovora survival during incompatible plant–pathogen interactions depends on the *rpoS* gene

Tobacco leaf sections inoculated with either of the assayed *E. amylovora* strains (CFBP 1430, *rpoS*, and *rpoS*$^+$) developed a typical HR within the following 20 h

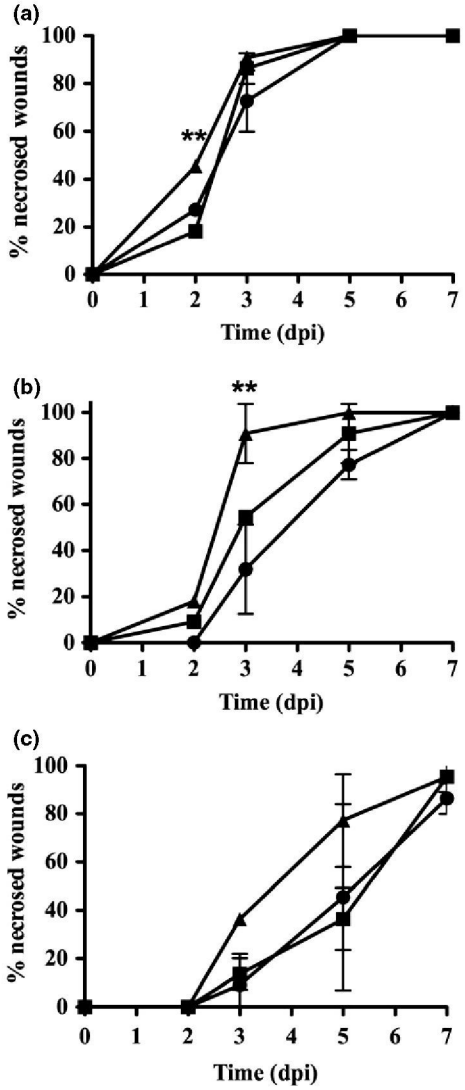

Fig. 5. Evaluation of virulence of the *Erwinia amylovora* strains CFBP 1430 (squares), *rpoS*$^-$ (triangles), and *rpoS*$^+$ (circles) in immature loquats at different inoculum doses. Fruits were inoculated in parallel with 10^4 (a), 10^3 (b), and 10^2 (c) CFU per wound. Represented data are the average values of two independent experiments with 11 replicates. Bars correspond to the SD. Asterisks denote statistically significant differences with the wild-type strain (**$P < 0.01$).

postinoculation (hpi), with no differences observed among strains. Negative controls did not elicit any response. With regard to survival inside tobacco leaf tissues (Fig. 7), all the strains behaved similarly during the first 24 hpi, showing a sharp loss of culturability from about 10^6 to 10^5 CFU cm^{-2}. During the following days, the wild-type strain first experienced a slight increase of culturability, followed by a slow drop of culturable cell numbers, which were about 10^5 CFU cm^{-2} at 8 dpi. This behavior was not observed in the mutant *rpoS*$^-$, which continued decreasing in culturable cell

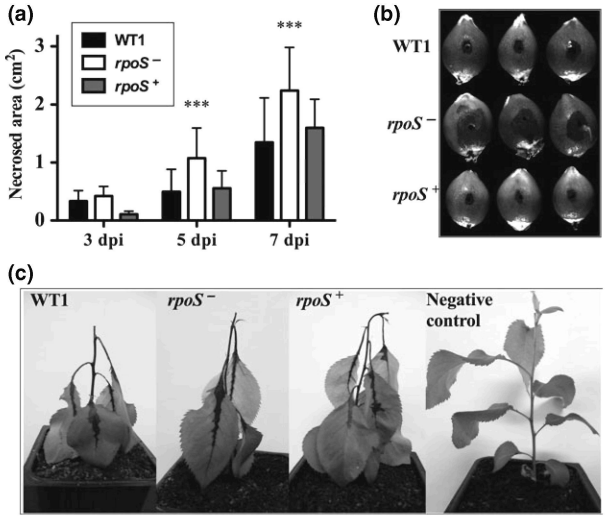

Fig. 6. Evaluation of the *Erwinia amylovora* virulence in immature fruits (a, b) and in pear plantlets (c) after their inoculation with 10^3 CFU per wound. Virulence was measured as the average necrosed areas in fruits or plants throughout time. Data in the graph are the average values of two independent experiments with 11 replicates. Bars correspond to the SD. Pictures represent characteristic fire blight symptoms in fruits and plantlets 5 days after their inoculation. WT1, *E. amylovora* strain CFBP 1430. Asterisks denote statistically significant differences with the wild-type strain (***$P < 0.001$).

numbers throughout the experimental period, reaching 6.5×10^3 CFU cm^{-2} at 8 dpi. The complemented strain behaved in a very similar way to the parental strain throughout the entire experiment. Differences between the mutant and the wild type (or the complemented strain) were statistically significant from time 2 ($P < 0.05$) to 8 dpi ($P < 0.001$).

Discussion

Microorganisms in nature are predominantly facing starvation and adapting to environmental fluctuations through complex developmental mechanisms tightly regulated at the genetic level (Ishihama, 1997; Morita, 1997; Edwards, 2000; Navarro Llorens *et al.*, 2010). In *E. coli* and other bacterial species, the alternative sigma factor RpoS plays an important role in the adaptation of the cell physiology to starvation, controlling genes related to stress responses (Edwards, 2000; Hengge-Aronis, 2000), DNA protection and repair (Vijayakumar *et al.*, 2004), nutrient recycling, and correct protein folding (Dong & Schellhorn, 2009), among others. In the present study, an *rpoS* mutant of the reference European *E. amylovora* strain CFBP 1430 was obtained, and the role of *rpoS* on starvation responses and other functions related to this gene hitherto unexplored in the fire blight pathogen were investigated.

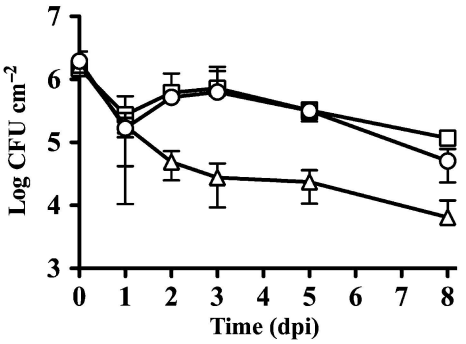

Fig. 7. Survival curves of *Erwinia amylovora* strains CFBP 1430 (squares), *rpoS*⁻ (triangles), and *rpoS*⁺ (circles) in tobacco leaf tissues. Represented data are the average values of two independent experiments performed in duplicate. Bars correspond to the SD.

Natural water microcosms were employed to analyze the role of the *rpoS* gene in *E. amylovora* responses to starvation, the main factor limiting bacterial survival in nature (Morita, 1997). The rapid entry of the *rpoS* mutant into the VBNC state accompanied by a loss of viability and cell integrity revealed the importance of this gene for the development of normal starvation responses in the fire blight pathogen, and hence for the survival and the adaptation of *E. amylovora* to natural oligotrophic conditions. These results were similar to those found in *E. coli* and *Salmonella enterica* mutants in the same gene (Boaretti *et al.*, 2003; Kusumoto *et al.*, 2012), indicating common functions of *rpoS* in human and plant pathogenic bacterial species. Regarding this, both survival under nutrient scarcity and the ability to form colonies on solid-rich media, a feature that is lost during entry into the VBNC state, have been related to the ability of bacterial cells to face the internal and external oxidative stresses generated by starvation-induced growth arrest and by growth on solid media, respectively (McDougald *et al.*, 2002; Boaretti *et al.*, 2003; Kong *et al.*, 2004). Interestingly, the sigma factor RpoS controls the expression of genes encoding catalases and many other proteins related to protection against oxidative stress (Eisenstark *et al.*, 1996; Hengge-Aronis, 2000, 2002). Accordingly, the altered regulation of oxidative stress-related proteins in the *rpoS* mutant might explain the altered starvation responses observed in this strain.

The alternative sigma factor RpoS controls genes related to stress resistance during the entry into stationary phase, and as a consequence, stationary phase cells become cross-protected against multiple stresses to which exponentially growing cells are sensitive (Hengge-Aronis, 2000; Lacour & Landini, 2004; Navarro Llorens *et al.*, 2010; Battesti *et al.*, 2011). In our work, we analyzed the role of *rpoS* on *E. amylovora* stationary phase cross-protection against oxidative and osmotic stresses, directly

related to the infectious process in the fire blight pathogen (Pusey, 2000; Venisse *et al.*, 2001), and also against acid stress, a condition to which *E. amylovora* and other phytopathogens have to adapt to colonize the plant apoplast (Grignon & Sentenac, 1991). The significant differences between the survivability of log and stationary phase cells of the parental and the *rpoS* mutant strains demonstrated the *rpoS* gene requirement for complete *E. amylovora* stationary phase cross-protection against oxidative, osmotic, and acid stresses. This highlights the contribution of *rpoS* to *E. amylovora* protection against these stresses during host infections and the apoplast colonization of nectaries or leaves. We additionally reported, for the first time, the essential role of the *rpoS* gene for *E. amylovora* stationary phase cell protection against heat shock, to which bacteria have to cope with environmental temperature fluctuations. This stress depends on the expression of heat shock proteins, which have been involved in the adaptation of bacterial cells to the environment (Feder & Hofmann, 1999). *E. amylovora,* usually detected in temperate regions, has, in recent years, been spreading to warmer countries which have more pronounced temperature variations (EPPO, 2013). Moreover, heat-shock proteins have also been implicated in infectious process in both animal and plant pathogens (Mantis & Winans, 1991; Feder & Hofmann, 1999), including *E. amylovora* during infection of immature fruits (Zhao *et al.*, 2005).

It is noteworthy that *rpoS* was apparently unnecessary for stationary phase cross-protection against acid shock. *E. amylovora* cells are subjected to this stress during fruit wound colonization, because of the release of tissue cell vacuolar compounds to the apoplast during the wounding, reaching pHs as low as 3.5 or 4. The colonization of wounds is a natural infection route frequently used by the fire blight pathogen (Vanneste & Eden-Green, 2000). The *rpoS*-independent protection of stationary phase cells against some stresses has been previously reported in *Vibrio vulnificus* (Rosche *et al.*, 2005) and *Pseudomonas aeruginosa* (Jørgensen *et al.*, 1999), and it has been attributed to other factors different to RpoS acting also during the entry into stationary phase.

The alternative sigma factor RpoS participates in the regulation of virulence in several pathogens (Dong & Schellhorn, 2010), including phytopathogenic bacteria (Flavier *et al.*, 1998; Andersson *et al.*, 1999; Wilf & Salmond, 2012). Consistently, we analyzed the participation of *rpoS* in the control of different virulence/pathogenicity factors such as motility and amylovoran and levan production. Motility has been reported as important during the early *E. amylovora* infection process, allowing cells to colonize apple blossoms, or to reach other natural infection sites such as stomata on the leaves (van der Zwet *et al.*, 2012). Amylovoran and levan are the two main exopolysaccharides of the fire blight pathogen. The first one is considered a pathogenicity factor, necessary for fire blight symptom development, and the second one is a virulence factor (Geider, 2000; van der Zwet *et al.*, 2012; Vrancken *et al.*, 2013). Both exopolysaccharides play additional roles, protecting *E. amylovora* cells against environmental challenges such as oxidative stress, desiccation, or starvation (Venisse *et al.*, 2001; Ordax *et al.*, 2010; Vrancken *et al.*, 2013). Our results revealed that *rpoS* contributes to the regulation of motility and the production of exopolysaccharides. In the case of motility, the *E. amylovora rpoS* mutant resulted significantly less motile than the parental strain, indicating the role of *rpoS* in enhancing cell motility during the colonization of nectaries and/or leave stomata under environmental conditions. Similar results were observed in *V. vulnificus* or *P. aeruginosa* (Dong & Schellhorn, 2010), although the control exerted by RpoS on motility-related genes may vary depending on the bacterial species analyzed (Dong & Schellhorn, 2010; Wilf & Salmond, 2012). With respect to the observed alteration of exopolysaccharide production in the *E. amylovora rpoS* mutant, mainly in levan secretion, a similar phenomenon has also been observed in *E. coli* (Ionescu & Belkin, 2009), *P. aeruginosa* (Suh *et al.*, 1999), and in the phytopathogenic bacterium *Ralstonia solanacearum* (Flavier *et al.*, 1998), but, as far as we know, this is the first report in the fire blight pathogen.

Given that the *E. amylovora rpoS* mutant showed an increased sensitivity to different stresses and an altered production of exopolysaccharides, the expected phenotype derived from the *rpoS* mutation would be an altered virulence, either diminished because of the sensitivity to stress inside the plant (Andersson *et al.*, 1999) or increased due to the overproduction of levan (Bereswill & Geider, 1997; Geider, 2000). According to our results, the *rpoS* mutation in the *E. amylovora* strain CFBP 1430 had different effects depending on the host model employed. In this respect, the mutant *rpoS*$^{-}$ was more virulent in immature loquats, but apparently as virulent as the parental strain in pear plantlets, indicating *rpoS* dispensability for fire blight symptom development, according to Anderson *et al.* (1998). However, there might be some kind of regulation of genes related to necrosis, at least in immature loquats, as the strain *rpoS*$^{-}$ caused more intense necrosis than the parental strain. Divergences in virulence results depending on the host assayed have also been reported in *rpoS* mutants of *Pectobacterium carotovorum* ssp. *carotovorum* (Andersson *et al.*, 1999) and *Serratia* sp. (Wilf & Salmond, 2012) and might be due to the distinct organs exposed to the pathogen, the inoculation procedure, and/or the different chemical composition or defenses elicited in each type of organ/host.

To explore the role of *rpoS* during *E. amylovora* incompatible interactions, we investigated the ability of the *rpoS* mutant to elicit a normal HR response and survive in tobacco plant tissues. The similar onset and characteristics of the necrosis observed in tobacco leaves inoculated either with the parental strain or the *rpoS* mutant revealed an apparently unaltered HR-inducing system. However, while the parental strain was able to slightly increase its numbers a few days after infiltration into tobacco leaves, the *rpoS* mutant showed a continuous drop of culturability over time. Differences among strains could be related to the inability of the mutant to face up to the stress conditions of the tobacco leaves' apoplast during the HR. This agrees with results of stationary phase cross-protection assays, in which *rpoS* was important for the protection of *E. amylovora* cells against oxidative stress. Interestingly, unlike other plant pathogens, *E. amylovora* also elicits an HR during compatible interactions (Venisse *et al.*, 2001), but the survival of the *rpoS* mutant was not compromised when artificially inoculated into immature loquats or pear plantlets. This apparent contradiction might be explained by the protective effect of the *E. amylovora* amylovoran capsule, whose production is induced by sorbitol and other environmental signals present in susceptible hosts (Geider, 2000), but not in non-*Rosaceae* plants (Deguchi *et al.*, 2006) during incompatible interactions.

In summary, this study reports new roles for the sigma factor RpoS in different ecological aspects of the fire blight pathogen life cycle. First, our results demonstrate the important contribution of the *rpoS* gene to the maintenance of culturability during the starvation survival response, as well as viability and cell integrity during entry into the VBNC state, revealing new aspects of these survival strategies not yet reported in any phytopathogenic bacteria. Second, *rpoS* is necessary for full stationary phase cross-protection against oxidative, osmotic, and acid stresses, and also against heat shock, which are directly related to the *E. amylovora* infectious process and/or survival in host and nonhost environments. Third, we provide evidence of the control exerted by the regulator RpoS on swimming motility and exopolysaccharide synthesis, as well as on fire blight symptom development in immature fruits, a trait not yet described in *E. amylovora*. Finally, we show, for the first time, that this sigma factor is essential for survival in the face of plant defense mechanisms during incompatible plant–pathogen interactions.

Acknowledgements

This research was supported through projects AGL2008-05723-C02-02, UV-INV-AE 112-66196, AGL2012-32516 and also a predoctoral fellowship from the Spanish 'Ministerio de Educación, Cultura y Deporte'. We thank C Navajo (SCSIE, Universitat de València, Spain) for the expert technical assistance on plant material management. We gratefully acknowledge MM López (Instituto Valenciano de Investigaciones Agrarias, Moncada, Spain) for kindly providing tobacco plants and also K Geider (Max-Planck-Institut für Zellbiologie, Rosenhof, Heidelberg) for making *E. amylovora* strains Ea 1/79, Amy$^-$, and Lev$^-$ available to us. We also are grateful to 'Speak English' (Valencia, Spain) and Daniel Sheerin for revising the English text.

Statement

We describe new roles for the *rpoS* gene in *E. amylovora*, including its contribution to: (1) the starvation survival and VBNC responses; (2) the stationary phase cross-protection; (3) the control of virulence factors such as motility and exopolysaccharide synthesis; (4) virulence; and (5) survival during incompatible plant–pathogen interactions.

References

Anderson M, Pollitt CE, Roberts IS & Eastgate JA (1998) Identification and characterization of the *E. amylovora rpoS* gene: RpoS is not involved in the induction of fireblight disease symptoms. *J Bacteriol* **180**: 6789–6792.

Andersson RA, Kõiv V, Norman-Setterblad C & Pirhonen M (1999) Role of RpoS in virulence and stress tolerance of the plant pathogen *Erwinia carotovora* subsp. *carotovora*. *Microbiology* **145**: 3547–3556.

Battesti A, Majdalani N & Gottesman S (2011) The RpoS-mediated general stress response in *Escherichia coli*. *Annu Rev Microbiol* **65**: 189–213.

Bereswill S & Geider K (1997) Characterization of the *rcsB* gene from *Erwinia amylovora* and its influence on exopolysaccharide synthesis and virulence of the fire blight pathogen. *J Bacteriol* **179**: 1354–1361.

Boaretti M, del Mar Lleo M, Bonato B, Signoretto C & Canepari P (2003) Involvement of *rpoS* in the survival of *Escherichia coli* in the viable but non-culturable state. *Environ Microbiol* **5**: 986–996.

Bugert P & Geider K (1995) Molecular analysis of the *ams* operon required for exopolysaccharide synthesis of *Erwinia amylovora*. *Mol Microbiol* **15**: 917–933.

Deguchi M, Bennett AB, Yamaki S, Yamada K, Kanahama K & Kanayama Y (2006) An engineered sorbitol cycle alters sugar composition, not growth, in transformed tobacco. *Plant Cell Environ* **29**: 1980–1988.

Dong T & Schellhorn HE (2009) Global effect of RpoS on gene expression in pathogenic *Escherichia coli* O157:H7 strain EDL933. *BMC Genom* **10**: 349.

Dong T & Schellhorn HE (2010) Role of RpoS in virulence of pathogens. *Infect Immun* **78**: 887–897.

Du Z & Geider K (2002) Characterization of an activator gene upstream of *lsc*, involved in levan synthesis of *Erwinia amylovora*. *Physiol Mol Plant Pathol* **60**: 9e17.

Edmunds AC, Castiblanco LF, Sundin GW & Waters CM (2013) Cyclic di-GMP modulates the disease progression of *Erwinia amylovora*. *J Bacteriol* **195**: 2155–2165.

Edwards C (2000) Problems posed by natural environments for monitoring microorganisms. *Mol Biotechnol* **15**: 211–223.

Eisenstark A, Calcutt MJ, Becker-Hapak M & Ivanova A (1996) Role of *Escherichia coli rpoS* and associated genes in defense against oxidative damage. *Free Radic Biol Med* **21**: 975–993.

EPPO, European and Mediterranean Plant Protection Organization (2013) Diagnostic protocol for regulated pests: *Erwinia amylovora*. EPPO Standards PM 7/20 (2). *EPPO Bull* **43**: 21–45.

Falkenstein H, Bellemann P, Walter S, Zeller W & Geider K (1988) Identification of *Erwinia amylovora*, the fireblight pathogen, by colony hybridization with DNA from plasmid pEA29. *Appl Environ Microbiol* **54**: 2798–2802.

Farkas Á, Orosz-Kovács Z & Bubán T (2004) Floral biological studies on pear cultivars in relation to fire blight susceptibility. *Int J Hortic Sci* **10**: 25–30.

Feder ME & Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu Rev Physiol* **61**: 243–282.

Fellay R, Frey J & Krisch H (1987) Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of gram-negative bacteria. *Gene* **52**: 147–154.

Flavier AB, Schell MA & Denny TP (1998) An RpoS (sigmaS) homologue regulates acylhomoserine lactone-dependent autoinduction in *Ralstonia solanacearum*. *Mol Microbiol* **28**: 475–486.

Geider K (2000) Exopolysaccharides of *Erwinia amylovora:* structure, biosynthesis, regulation, role in pathogenicity of amylovoran and levan. *Fire Blight: The Disease and its Causative Agent, Erwinia amylovora* (Vanneste JL, Ed), pp. 117–140. CAB International, Wallingford, UK.

Glasner JD, Liss P, Plunkett G *et al.* (2003) ASAP, a systematic annotation package for community analysis of genomes. *Nucleic Acids Res* **31**: 147–151.

Gorris MT, Cambra E, Paulin JP, Chartier R, Cambra M & López MM (1996) Production and characterization of monoclonal antibodies specific for *Erwinia amylovora* and their use in different serological techniques. *Acta Hortic* **411**: 47–51.

Grignon C & Sentenac H (1991) pH and ionic conditions in the apoplast. *Annu Rev Plant Physiol* **42**: 103–128.

Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**: 557–580.

Hengge-Aronis R (2000) The general stress response in *Escherichia coli*. *Bacterial Stress Responses* (Storz G & Hengge-Aronis R, eds), pp. 161–178. American Society for Microbiology, Washington, DC.

Hengge-Aronis R (2002) Signal transduction and regulatory mechanisms in the control of the σS (RpoS) subunit of RNA polymerase. *Microbiol Mol Biol Rev* **66**: 373–395.

Ionescu M & Belkin S (2009) Overproduction of exopolysaccharides by an *Escherichia coli* K-12 *rpoS* mutant in response to osmotic stress. *Appl Environ Microbiol* **75**: 483–492.

Ishihama A (1997) Adaptation of gene expression in stationary phase bacteria. *Curr Opin Genet Dev* **7**: 582–588.

Ishimaru C & Klos EJ (1984) New medium for detecting *Erwinia amylovora* and its use in epidemiological studies. *Phytopathology* **74**: 1342–1345.

Jørgensen F, Bally M, Chapon-Herve V, Michel G, Lazdunski A, Williams P & Stewart GS (1999) RpoS-dependent stress tolerance in *Pseudomonas aeruginosa*. *Microbiology* **145**: 835–844.

Kamber T, Smits THM, Rezzonico F & Duffy B (2012) Genomics and current genetic understanding of *Erwinia amylovora* and the fire blight antagonist *Pantoea vagans*. *Trees* **26**: 227–238.

Konarska A, Masierowska M & Weryszko-Chmielewska E (2005) The structure of nectaries and nectar secretion in Common pear (*Pyrus communis* L.). *J Apic Sci* **49**: 85–92.

Kong I, Bates T, Hulsmann A, Hassan H, Smith B & Oliver JD (2004) Role of catalase and oxyR in the viable but nonculturable state of *Vibrio vulnificus*. *FEMS Microbiol Ecol* **50**: 133–142.

Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM 2nd & Peterson KM (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**: 175–176.

Kusumoto A, Asakura H & Kawamoto K (2012) General stress sigma factor RpoS influences time required to enter the viable but non-culturable state in *Salmonella enterica*. *Microbiol Immunol* **56**: 228–237.

Lacour S & Landini P (2004) SigmaS-dependent gene expression at the onset of stationary phase in *Escherichia coli*: function of sigmaS-dependent genes and identification of their promoter sequences. *J Bacteriol* **186**: 7186–7195.

Mantis NJ & Winans SC (1991) Characterization of the *Agrobacterium tumefaciens* heat shock response: evidence for a sigma 32-like sigma factor. *J Bacteriol* **174**: 991–997.

McDougald D, Gong L, Srinivasan S, Hild E, Thompson L, Takayama K, Rice SA & Kjelleberg S (2002) Defences against oxidative stress during starvation in bacteria. *Antonie van Leeuwenhoek* **81**: 3–13.

Morita RY (1997) *Bacteria in Oligotrophic Environments*. Starvation-Survival Lifestyle. Chapman-Hall, International Thomson Publishing, New York, NY.

Navarro Llorens JM, Tormo A & Martínez-García E (2010) Stationary phase in gram-negative bacteria. *FEMS Microbiol Rev* **34**: 476–495.

Ordax M, Marco-Noales E, López MM & Biosca EG (2010) Exopolysaccharides favor the survival of *Erwinia amylovora* under copper stress through different strategies. *Res Microbiol* **161**: 549–555.

Paulin JP & Samson R (1973) Le feu bactérien en France. II.—caractères des souches d' *Erwinia amylovora* (Burril) Winslow et al. 1920, isolées du foyer franco-belge. *Ann Phytopathol* **5**: 389–397.

Pusey PL (2000) The role of water in epiphytic colonization and infection of pomaceous flowers by *Erwinia amylovora*. *Phytopathology* **90**: 1352–1357.

Raymundo AK & Ries SM (1981) Motility of *Erwinia amylovora*. *Phytopathology* **71**: 45–49.

Roeder DL & Collmer A (1985) Marker-exchange mutagenesis of a pectate lyase isozyme gene in *Erwinia chrysanthemi*. *J Bacteriol* **164**: 51–56.

Rosche TM, Smith DJ, Parker EE & Oliver JD (2005) RpoS involvement and requirement for exogenous nutrient for osmotically induced cross protection in *Vibrio vulnificus*. *FEMS Microbiol Ecol* **53**: 455–462.

Santander RD, Català-Senent JF, Marco-Noales E & Biosca EG (2012) In planta recovery of *Erwinia amylovora* viable but nonculturable cells. *Trees* **26**: 75–82.

Santander RD, Oliver JD & Biosca EG (2014) Cellular, physiological and molecular adaptive responses of *Erwinia amylovora* to starvation. *FEMS Microbiol Ecol* **88**: 258–271.

Schneider CA, Rasband WS & Eliceiri KW (2012) NIH image to IMAGEJ: 25 years of image analysis. *Nat Methods* **9**: 671–675.

Smith HL Jr & Goodner K (1958) Detection of bacterial gelatinases by gelatin agar plate methods. *J Bacteriol* **76**: 662–665.

Smits THM, Rezzonico F, Kamber T, Blom J, Goesmann A, Frey JE & Duffy B (2010) Complete genome sequence of the fire blight bacterium *Erwinia amylovora* CFBP 1430 and comparison to other *Erwinia* spp. *Mol Plant Microbe Interact* **23**: 384–393.

Spinelli F, Vanneste JL, Marcazzan GL, Sabatini AG & Costa G (2005) Effect of prohexadione-calcium on nectar composition of pomaceous flowers and on bacterial growth. *NZ Plant Prot* **58**: 106–111.

Starr MP & Mandel M (1950) The nutrition of phytopathogenic bacteria IV. Minimal nutritive requirements of the genus *Erwinia*. *J Bacteriol* **60**: 669–672.

Suh SJ, Silo-Suh L, Woods DE, Hassett DJ, West SE & Ohman DE (1999) Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. *J Bacteriol* **181**: 3890–3897.

Thomson SV (2000) Epidemiology of fire blight. *Fire Blight: The Disease and its Causative Agent, Erwinia amylovora* (Vanneste JL, Ed), pp. 9–36. CAB International, Wallingford, UK.

Torriani A (1960) Influence of inorganic phosphate in the formation of phosphatases by *Escherichia coli*. *Biochim Biophys Acta* **38**: 460–479.

van der Zwet T, Orolaza-Halbrendt N & Zeller W (2012) *Fire Blight: History, Biology, and Management*. APS Press, St. Paul, MN.

Vanneste JL & Eden-Green S (2000) Migration of *Erwinia amylovora* in host plant tissue. *Fire Blight: The Disease and its Causative Agent, Erwinia amylovora* (Vanneste JL, Ed), pp. 73–83. CAB International, Wallingford, UK.

Venisse JS, Gullner G & Brisset MN (2001) Evidence for the involvement of an oxidative stress in the initiation of infection of pear by *Erwinia amylovora*. *Plant Physiol* **125**: 2164–2172.

Vijayakumar SR, Kirchhof MG, Patten CL & Schellhorn HE (2004) RpoS-regulated genes of *Escherichia coli* identified by random lacZ fusion mutagenesis. *J Bacteriol* **186**: 8499–8507.

Vrancken K, Holtappels M, Schoofs H, Deckers T & Valcke R (2013) Pathogenicity and infection strategies of the fire blight pathogen *Erwinia amylovora* in *Rosaceae*: state of the art. *Microbiology* **159**: 823–832.

Wilf NM & Salmond GP (2012) The stationary phase sigma factor, RpoS, regulates the production of a carbapenem antibiotic, a bioactive prodigiosin and virulence in the enterobacterial pathogen *Serratia* sp. ATCC 39006. *Microbiology* **158**: 648–658.

Zhao Y, Blumer SE & Sundin GW (2005) Identification of *Erwinia amylovora* genes induced during infection of immature pear tissue. *J Bacteriol* **187**: 8088–8103.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. PCR and RT-PCR confirmation of the *rpoS* mutation and complementation in *Erwinia amylovora*.

Fig. S2. Phenotypic confirmation of the *Erwinia amylovora rpoS* mutation and complementation, by the growth inhibition halo assay, using 25 mM H_2O_2.

Fig. S3. Gelatin hydrolysis assay using nutrient broth agar containing 4 % (w/v) gelatin, after an incubation period of 11 days at 28 °C. Gelatin hydrolysis appears as a whitish halo surrounding the bacterial colonies.